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The Potential Role of Integrin Receptor Subunits in the Formation of Local Recurrence and Distant Metastasis by Mouse Breast Cancer Cells

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Background: The mechanisms by which surgical injury fosters tumor growth are examined.

Methods: TA3Ha mouse breast tumor line and its subline (TA3AD) differing in their metastatic abilities as tested by two models were used. In model a, TA3Ha/TA3AD tumors were grown in the mammary fat pads of mice and then surgically removed with a curative intent. In model b, TA3Ha/TA3AD cells were injected intravenously into mice subjected to liver or spleen wedge resection. Frequency of tumor formation at various sites was assessed. Expression of integrin, immunoglobulin, and proteoglycan cell adhesion receptors on TA3Ha and TA3AD cells was examined by flow cytometry. The roles of these receptors in metastasis were examined by blocking them by selected ligands and/or antibodies.

Results: Frequencies of local recurrence and axillary metastasis after surgical resection, were 43% (32/74), and 37% (27/74) with TA3Ha tumors and 4% (1/29) at both sites with TA3AD tumors. Tumors at surgically injured spleen and the liver were seen in 75% (141/189) and 45% (107/240) of the mice with TA3Ha cells and in 8% (3/38) and 10% (4/42) of the mice with TA3AD cells. α_5 and CD44 receptors were expressed by TA3Ha cells but not by TA3AD cells. Other receptors examined were similarly expressed by both cell lines. Blocking of α_5 receptor by fibronectin reduced tumor implantation in a dose-dependent manner.

Conclusions: The data suggest a correlation among the ability to implant at surgically injured sites, to form local recurrence, and to express the fibronectin receptor subunit.

KEY WORDS: **breast cancer, injury, local recurrence, metastasis, integrins, fibronectin, extracellular adhesion molecules**

INTRODUCTION

Tumor cells have a proclivity to implant and grow at sites of injury and inflammation [1–11]. In some instances, this phenomenon is suspected to lead to clinical complications including the development of distant metastases [12–18], local recurrence [19,20], and recurrence at port sites after laparoscopic surgery [18]. If tumor implantation at wound sites is indeed related to these clinical manifestations, it is important to determine the significance and mechanisms by which tumor cells implant at wound sites. The approach taken in this study is to characterize tumor populations that differ in their ability to implant at surgical wound sites.

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Previous studies from our laboratory have shown that the TA3Ha mouse mammary adenocarcinoma cells when injected intravenously into syngeneic mice avidly implant at surgically injured liver, kidney, cecum, spleen, and the pelvic bone [3,21–32]. Furthermore, a subpopulation, TA3AD, derived from the parental TA3Ha cell line fails to implant in injured pelvic bone [23]. In the present studies, these two cell populations are used to examine the following: Are the TA3AD cells that fail to implant at injured pelvic bone also deficient in implanting in other organs subjected to surgical injury? Does the ability to implant at surgically injured sites correlate with the ability to form spontaneous metastasis from an orthotopic site (mammary fat pad) and to form local recurrence following "curative" surgical excision? Do the tumor cells use similar mechanisms to implant at healing wounds in different organ sites? In order to understand the mechanisms by which tumor cells implant at surgically injured sites, TA3Ha and TA3AD cells have been characterized for the expression of a panel of cell surface receptors involved in cell–extracellular matrix protein interactions at the wound sites. Another aspect examined is whether the same ligands that block tumor implantation at injured liver site by interacting with the cell adhesion receptors also block tumor implantation at other sites of surgery.

MATERIALS AND METHODS

Mice

Strain A female mice were obtained from the National Cancer Institute (Frederick Cancer Research Facility, Frederick, MD). These mice were young adult females weighing an average of 20 g at the beginning of each experiment. Mice were acclimatized to the Evanston Hospital animal care facility for 3 or more days prior to use. All animal experiments were reviewed and approved by the Evanston Hospital Institutional Animal Care and Usage Committee.

Reagents

Nutrient medium L-15, HBSS (Hanks' balanced salt solution), trypsin, and fetal bovine serum (FBS) were purchased from the Central Facilities, Northwestern University Cancer Center (Chicago, IL).

Tumor Cells

Spontaneous mammary adenocarcinoma line TA3Ha [24] and its subclone TA3AD [23] syngeneic to Strain A mice were used. Both cell types form solid tumors upon subcutaneous inoculation, lung colonies upon intravenous inoculation, and hemorrhagic ascites tumor upon intraperitoneal injection [3,25,26]. For each experiment, ascites cells were freshly harvested, washed in HBSS, and suspended in L-15 medium containing 10% FBS. In most experiments, 10^5 cells in 0.05 ml were injected intravenously into each mouse. To obtain solid tumors, 10^5 cells were injected into the mammary fat pad of mice. By day 7, the resulting TA3Ha tumors reached an average geometric mean diameter (GMD) of 0.62 ± 0.05 cm and TA3AD a GMD of 0.67 ± 0.24 cm. These two tumor lines have been tested in a hematogenous metastasis model to compare their efficacies to implant at surgically injured spleen and liver. Second, these tumor lines were tested in an orthotopic tumor model to assess their abilities to form spontaneous metastasis and local recurrence.

Model of Hematogenous Metastasis at Surgically Injured Sites

In this model, the spleen, cecum, or the liver of syngeneic mice were subjected to surgical injury. Following surgery, the mice were injected intravenously with TA3Ha or TA3AD cells at various times. The surgical procedure for the liver has been described earlier [27] and the procedures for the spleen and cecum are presented below.

Spleen. Mice were anesthetized by intraperitoneal injection of 70 mg/kg pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL). Skin over the abdomen was prepared by applying 70% ethanol and betadine. An incision (0.5–1.0 cm) was made in the left subcostal area through the skin and peritoneum. The spleen was exteriorized and about 20% of it was excised from the lower pole using the MACAN MV-8 electrosurgical unit (MACAN, Chicago, IL) and Lectrode blade (Valleylab, Boulder, CO). The spleen was replaced into the peritoneal cavity. Skin and peritoneal wounds were closed in a single layer using 5.0 polyglactin suture.

Cecum. Mice were anesthetized and prepared as above. Incision in the skin and peritoneum was made in the lower left abdominal site. The cecum was exteriorized and a 0.5-cm enterotomy performed. The incisional wound was closed using 7.0 prolene suture and cleaned with 70% alcohol. The cecum was replaced back into the peritoneal cavity. The skin and peritoneal wounds were closed as above.

Spontaneous Metastasis and Local Recurrence Models

These models have been previously described by us [28]. In brief, 10^5 TA3Ha or TA3AD cells were injected into the mammary fat pads of syngeneic mice. Solid tumors begin to appear in about 4 days. By day 7, the TA3Ha and TA3AD tumors measure 0.62 ± 0.05 and 0.67 ± 0.24 cm (geometric mean diameter), respectively. Untreated, these tumors continue to increase in size at a rate of 0.159 ± 0.06 cm/day. Both TA3Ha and TA3AD tumors locally extend into the peritoneum and lead to the formation of lethal hemorrhagic ascites tumors. There is no significant difference in the survival periods of mice bearing TA3Ha and TA3AD tumors.

To assess local recurrence and distant metastasis after surgical treatment, TA3Ha or TA3AD tumors grown in

the mammary fat pads for 7 days were surgically excised with a "curative" intent; i.e., all macroscopic tumor visible under 4X magnification was removed with some surrounding normal tissue, to ensure that no gross tumor is left behind. Following the surgical removal of the tumor, the skin wound was closed using 5.0 polyglactin suture. The mice were monitored for varying periods of time. The experiments were terminated at predetermined times by euthanizing the mice by intraperitoneal injection of 200 mg/kg pentobarbital. All mice were subjected to complete autopsy examination.

Identification of the Cell Surface Receptors of Relevance to Tumor Implantation at Surgically Injured Sites

TA3Ha and TA3AD cell populations were compared for the expression of a panel of cell adhesion receptors using specific monoclonal antibodies and flow cytometric method. The reagents used were: Anti-mouse monoclonal antibodies CD61 (β_3-integrin; clone 2C9.G2), CD29 (β_1-integrin; clone 9EG7), CD31 (PECAM-1; clone MEC 13.3), CD49d (α_4-integrin; clone R1-2), CD49e (α_5-integrin; clone 5H10-27/MFRS), CD51 (α_v integrin; clone H9.2B8), CD44 (hyaluronic acid: clone IM7), and isotype control antibodies. These reagents were purchased from PharMingen (San Diego, CA). Antibodies against CD49f (α_6 integrin; clone GoH3) were obtained from Amac (Westbrook, ME).

Flow Cytometric Methods

Our routine procedure has been published [29]. Briefly, TA3Ha or TA3AD ascites cells were washed in cold FACS-PBS (phosphate-buffered saline containing 5% FBS and 0.02% sodium azide) and incubated with 0.5–1 μg primary antibodies. After incubation, cells were washed three times and then incubated with a secondary antibody conjugated with an appropriate fluorochrome. Cells were washed and fixed in 2% paraformaldehyde. The staining pattern was analyzed using the Beckton-Dickinson FACScan and Consort-30 and Lysys software. Background staining from control cells processed without the specific primary antibodies and from those treated with isotype controls was run with each test antibody sample. The background is shown as the solid black portion in the histograms.

Identification of the Cell Adhesive Matrix Proteins Involved in Tumor Implantation at Surgically Injured Sites

To understand whether the cell adhesion receptors uniquely expressed TA3Ha cells (the cells that avidly implant at wound sites) have a role in implantation at sites of surgical injury, these cells were pretreated with ligands known to occupy cell adhesion receptors as described previously [30,31]. Cells were suspended in HBSS containing different concentrations of cell attachment proteins or peptides, disodium EDTA (ethylene diamine tetraacetate), or monoclonal antibodies. Mouse cellular fibronectin was from Fibrogenex (Chicago, IL). Preparation and properties of this fibronectin have been published elsewhere [30]. Human serum fibronectin (>93% pure) was a gift from Dr. Chin Huang, (Armour Pharmaceuticals, Kankakee, IL). Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro (RGDSPASSKP: purity 98%), and Arg-Gly-Asp-Ser (RGDS: purity 98%) were purchased from Sigma Chemical Company (St. Louis, MO). Bovine fibrinogen (99% clottable) was from Miles Scientific (Naperville, IL). Mouse laminin (purity ≥90%) was from Boehringer Mannheim Biochemicals (Indianapolis, IN).

The cells were treated with the above reagents for 60 min at 37°C. Treated and control cells were injected intravenously into each mouse. In most of the experiments, 10^5 cells were injected in 0.05 mL incubation mixture. In most experiments, mice were autopsied 14 days after tumor injection. Routinely, macroscopic examination was performed on all organs except the brain. Representative tissues were processed for microscopic examination.

RESULTS

Implantation of Intravenously Injected TA3Ha and TA3AD Cells at Sites of Surgical Trauma

Neither TA3Ha nor TA3AD cells, when injected intravenously, formed tumor in the uninjured spleen or the liver of syngeneic mice. However, when these organs were surgically injured, 75% ($P < 0.001$) of the mice injected with 10^5 TA3Ha cells developed tumor in the spleen wound. Tumor implantation increased directly with the cell inoculum (Table I). A parallel increase of tumor formation was seen in the lung ($r = 0.999$), surgically injured skin ($r = 0.959$), and surgically injured peritoneum ($r = 0.984$). In striking contrast, the TA3AD cells (10^5) formed tumors at the surgically injured spleen in significantly fewer mice (8%, $P < 0.00001$) compared to the TA3Ha cells. When the cell inoculum was increased to 5×10^5, and 10×10^5, the frequency of tumor implantation at the surgically injured spleen increased to 10%, and 29%, respectively. The differences in the frequency of tumor formation between TA3Ha and TA3AD for corresponding cell numbers are again significant ($P < 0.002$). Based on the number of cells injected and the frequency of tumor implantation, the TA3AD cells are calculated to be at least 30 times less efficient than TA3Ha cells. These differences are reproduced when tested in the hepatic wound model (Table I). While 45% of the mice formed TA3Ha tumor in the hepatic wound, only 10% of the mice injected with TA3AD formed such tumors. Tumor formation at skin or peritoneal wounds was also less efficient with TA3AD cells when compared with the TA3Ha cells. This inability to implant at wound

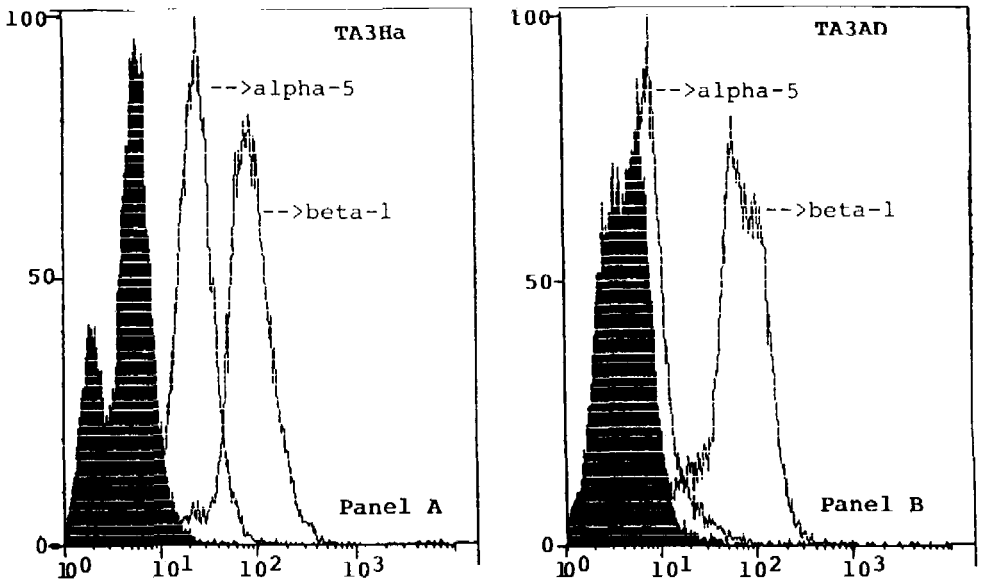

Fig. 1. Histogram representing the expression of α_5 and β_1 integrin subunits on TA3Ha cells (**A**) and on TA3AD cells (**B**). Solid section represents the background staining (control). X-axis: relative fluorescence intensity; Y-axis: relative number of cells.

TABLE I. Implantation of TA3Ha and TA3AD Cells at Sites of Surgical Injury in Mice

			No. of mice with tumor (%)					
Cell type	No. of cells	No. of mice	Lung	Surg. skin	Surg. peritoneum	Surg. spleen	p*	Tumor at any site
TA3Ha	10^5	189	108 (57)	10 (5)	39 (21)	141 (75)		174 (92)
TA3AD	10^5	38	25 (66)	1 (3)	1 (3)	3 (8)	<0.000001	29 (76)
TA3Ha	5×10^5	10	7 (70)	1 (10)	3 (30)	9 (90)		10 (100)
TA3AD	5×10^5	10	10 (100)	1 (10)	1 (10)	1 (10)	<0.000001	10 (100)
TA3Ha	10×10^5	18	16 (89)	5 (28)	9 (50)	17 (94)		18 (100)
TA3AD	10×10^5	7	7 (100)	0 (0)	0 (0)	2 (29)	<0.002	7 (100)
						Surg liver		
TA3Ha	10^5	240	129 (54)	23 (10)	76 (31)	107 (45)		208 (87)
TA3AD	10^5	42	25 (40)	7 (16)	12 (29)	4 (10)	<0.000001	29 (69)

Controls (TA3Ha 10^5 cells and TA3AD 10^5 cells) represent both historical as well as concurrent controls. They are presented together as the results were reproducible. TA3Ha: r = 0.999 for cell number vs. % tumor take in the lung; r = 0.959 for cell number vs. % tumor take at skin site; r = 0.984 for cell number vs. % tumor take at peritoneal site; r = 0.922 for cell number vs. % tumor take at spleen site; TA3AD: r = 0.832 for cell number vs. % tumor take in the lung; no correlation for cell number vs. % tumor take at skin or peritoneum; r = 0.875 for cell number vs. % tumor take at spleen site.

r = correlation ceofficient.

* P value of the difference between TA3Ha and TA3AD determined by Chi-square test.

sites by TA3AD is not because these cells are nontumorigenic, for they were extremely proficient in forming lung colonies upon intravenous injection (Table I). These results confirm and expand our earlier findings that the TA3AD cells are deficient in their ability to implant at surgical wound sites following intravenous injections.

Formation of Spontaneous Metastasis and Recurrence After Surgical Excision of Orthotopically Grown Tumor

Every mouse inoculated with 10^5 TA3Ha or TA3AD cells into the mammary fat pad developed local solid tumor. By day 7, the average GMD of TA3Ha tumor was 0.62 ± 0.05 cm and that of TA3AD was 0.67 ± 0.24 cm. Untreated, both tumor types locally extended into the peritoneum and resulted in ascites tumor by 3 weeks after tumor inoculation. The mice were moribund at this time and they were euthanized. Autopsy examination showed that the TA3Ha tumor-bearing mice had spontaneous metastasis in the ipsilateral axilla and the lungs in 44% (13/30) and 33% (10/30) of cases, respectively. In contrast, none of the 24 mice inoculated similarly with TA3AD tumor cells developed metastasis in either the axilla or the lung.

TABLE II. Frequency of Local Recurrence and Distant Metastasis by TA3Ha and TA3AD Tumors After Surgical Excision

		Mice with tumor							
		Local failure		Axillary metastasis		Lung metastasis		Disease free	
Tumor	No. of mice	n	%	n	%	n	%	n	%
TA3Ha	74	32	(43)	27	(37)	12	(16)	17	(23)
TA3AD	29	1	(4)	1	(4)	0	(0)	27	(93)

To examine the frequency of local recurrence (relapse at the site of original tumor excision), the tumors grown orthotopically were excised 7 days after tumor injection. TA3Ha and TA3AD tumors were comparable in size at this time (as noted above). Upon excision of these tumors, the mice bearing TA3Ha tumor developed recurrence at the local site, ipsilateral axillary area, and in the lungs at frequencies of 43%, 37%, and 16%, respectively (Table II). TA3AD tumor formed recurrence at these locations in 4%, 4%, and 0% of the mice, respectively. Furthermore, at the time of autopsy, 23% of the mice that had TA3Ha tumor were disease-free, whereas, 93% of the mice that had their TA3AD tumor excised, were tumor-free (Table II).

These results demonstrate that the TA3Ha cells which avidly implant at surgical sites are also proficient in forming spontaneous metastasis from the orthotopic site and in forming local recurrence and distant metastasis. In comparison, the TA3AD cells that are deficient in wound implantation are also deficient in their ability to form metastasis. It is noteworthy that the TA3AD cells are able to form lung colonies upon intravenous injection and solid tumor growth at the orthotopic site as efficiently as the TA3Ha cells. Thus, all three in vivo assays evaluating hematogenous metastasis to the injured sites, spontaneous metastasis, and local recurrence clearly demonstrate that the TA3AD cells lack the ability to form metastasis. Consequently, this pair of tumor lines offer an exciting possibility to probe into the mechanisms of tumor implantation at surgical sites. As a first step, we characterized the two cell populations for the expression of a selected panel of cell surface receptors that are likely to influence tumor cell–matrix protein interaction.

Cell Adhesion Receptor Expression

Flow cytometric analysis of the cell surface receptors revealed that the TA3Ha cells expressed α_5 (fibronectin receptor subunit), α_6 (laminin receptor subunit), and β_1 (which associates with several α-subunits) and CD44 receptor. α_4 (Alternative fibronectin receptor subunit), α_v (vitronectin receptor subunit), β_3 (which associates with several α-subunits), CD54 (ICAM; intercellular adhesion molecule), or CD31 (PECAM; platelet endothelial cell adhesion molecule) was not detectable with the monoclonal antibodies used (Table III). TA3AD cells showed β_1 and α_6 integrin subunits and a weak expression of CD44. β_3, α_4, α_5, α_v, CD54, or CD31 was not detectable. Figure 1 represents the patterns of α_5 and β_1 expression in TA3Ha and TA3AD cells. The major difference between the TA3Ha and TA3AD cell types as regards the receptors examined is seen only in the expression of the classical fibronectin receptor α_5 subunit (Table III). The α_5 receptor subunit, in association with the β_1 subunit recognizes and binds RGDS peptides and fibronectin. Fibronectin binding to TA3Ha cells has been confirmed in an earlier study by us [30]. It must however be noted that the RGD peptides can associate with cells in an integrin independent manner also [32].

Identification of the Ligands Relevant to Tumor Implantation at Wound Sites

If the α_5 receptor has importance in tumor implantation, blockade of this receptor by pretreatment of TA3Ha cells with RGDS containing proteins and peptides, or anti-α_5-blocking antibodies should also block the cells from implanting at surgical sites. Furthermore, since the function of the integrin receptors is sensitive to cations [33], pretreatment with EDTA is expected to inhibit tumor implantation. Such studies would then allow us to identify the ligands that may determine tumor implantation in the healing wounds. The results are summarized in Table IV. Pretreatment with human serum fibronectin or mouse cellular fibronectin reduced the formation of splenic wound tumors significantly ($P < 0.005$). Similarly, RGDS and RGDSPASSKP peptides showed significant tumor inhibitory effects ($P < 0.005$) at $\geq$200 μg/ml. Laminin, an important component of the basement membrane, at 100 or 200 μg/ml also reduced tumor implantation to 2/9 (22%) and 2/10 (20%; $P < 0.005$). Considering that the TA3Ha cells express α_6-integrin subunit, it is not surprising that laminin had tumor blocking effects. However, the non-implanting TA3AD cells also expressed α_6-receptor subunit. Thus, α_6 expression may be important but not sufficient for determining tumor implantation at surgical sites. Pretreatment of the cells with anti-α_5 partial blocking antibodies, reduced TA3Ha tumor implantation from 75% to 50% (Table IV). This inhibition is only partial and not considered statistically significant. Similar treatment with iso-

TABLE III. Expression of Cell Surface Receptors Relevant to Attachment at Wound Sites

	Receptor subunits									
Cells	α_4	α_5	α_6	α_v	β_1	β_3	CD44	CD54	CD31	Metastasis
TA3Ha	Neg	Pos	Pos	Neg	Pos	Neg	Pos	Neg	Neg	Yes
TA3AD	Neg	Neg	Pos	Neg	Pos	Neg	±	Neg	Neg	No

type matched antibodies against CD31 (PECAM), a receptor not expressed by TA3Ha resulted in no tumor inhibition. EDTA, which disrupts the functional association of α/β-subunits of integrin receptors, inhibits tumor implantation significantly (Table IV). This finding further supports the contention that the integrin receptors are involved in implantation of tumors at surgical sites. In this study, fibrinogen exhibited a modest inhibitory effect ($P < 0.05$) at 400μg/ml. The synthetic peptide fragment of fibrinogen, namely KQAGDV (Lys-Gln-Ala-Gly-Asp-Val), showed no significant inhibitory effects. Similarly, treatment with albumin and RGES (Arg-Gly-Glu-Ser) peptide had no tumor inhibitory effects (data not shown).

In order to examine whether the tumor inhibitory effects of fibronectin or its synthetic peptide fragment RGDSPASSKP are due to cytotoxicity, the following experiments were conducted. TA3Ha cells treated with human fibronectin (200 μg/ml), or mouse fibronectin (100 or 200 μg/ml) for 60 min at 37°C were injected intraperitoneally into mice without surgery. All these mice developed hemorrhagic ascites tumor and died in 9–10 days. These results are similar to those by the untreated control cells. Similar results were obtained with TA3Ha cells treated with 100μg/ml RGDSPASSKP. In an earlier study, we found that fibronectin does not affect the TA3Ha proliferative activities [30]. Thus, it is likely that tumor inhibition is due to occupancy of relevant cell surface receptors and consequent hindering of cells from attaching to the natural ECM components.

The tumor inhibition results seen in this study are comparable to those found in the case of TA3Ha implantation at surgically induced liver wounds [30,31]. Thus, it is likely that the TA3Ha cells use similar mechanisms to implant at surgical sites in different organs. To examine the possibility further, the frequency of tumor implantation in relation to the timing of surgery and cell injection was examined. In these experiments, mice were subjected to surgical procedures in the spleen and cecum. These mice were injected intravenously with 10^5 TA3Ha cells at various periods after surgery. The results are compared with those from similar earlier experiments using injured liver as the target. As the results in Table V show, tumor implantation was the most efficient (75%) when the tumor cells were injected immediately after spleen surgery. As the tumor injection was delayed by 1, 4, 11, or 15 days postsurgery, tumor formation decreased to 25%, 8%, 0%, and 0%, respectively. Similarly, tumor implantation in the cecum wound declined from 75% to 10%, 29%, and 11% as the interval between surgery and tumor injection was increased from day 0 to postsurgery day 3, 8, or 10, respectively. This pattern is practically identical to that found in the case of the liver (Table V) indicating that the TA3Ha cells are similarly influenced by factors released from fresh wounds in different organs.

DISCUSSION

The data demonstrate that TA3Ha cells implant in the surgically injured spleen and liver in a high proportion of mice. By contrast, the TA3AD variant derived from TA3Ha is extremely inefficient in implanting at corresponding sites. Our earlier studies showed that the TA3AD cells are also deficient in their ability to implant at surgically injured pelvic bone [23]. These findings demonstrate that the ability or the lack of it to implant at surgical sites applies across different organs. Thus, for the first time, a system in which variants derived from the same tumor that differ in their ability to implant at surgical sites has become available. This system offers an interesting opportunity to correlate the ability to implant at wound sites and to form local and distant relapse. Likewise, it gives an opportunity to probe into the mechanisms by which tumor cells implant at wound sites.

Both TA3Ha and TA3AD cells formed solid tumors when injected into the mammary fat pad of mice with equal efficiency. Upon surgical excision of the TA3Ha tumor, local recurrence (recurrence at the surgical scar) was evident in 43% of the mice. Some of these mice as well as others also showed distant metastasis so that only 23% of the mice remained tumor free at autopsy. In striking contrast, TA3AD tumors of comparable size, when surgically excised, formed local recurrence in 4% of cases and distant metastasis as well in 4% of the mice. Ninety-three percent of the mice were tumor free at autopsy (some were autopsied after 350 days). Thus, although TA3AD cells grow aggressively in syngeneic mice, they fail to form local recurrence or distant metastasis after surgical excision of the primary tumor.

The precise mechanisms by which primary tumor excision leads to local recurrence is not known but the present data show an intriguing relationship between the ability to implant at wound sites and to form recurrent tumors. One possibility is that the cells disseminated at the surgery or prior to it, may return to the surgical wound and implant there. The factors that drive the cells to return to the

TABLE IV. Effects of RGDS-Containing Proteins and Peptides on Wound Implantation by TA3Ha Cells

Concen. (μg/ml)	No. of mice	Mice with tumor: Lung n	Lung %	Lung P	Surg. spleen n	Surg. spleen %	Surg. spleen P	Tumor any site n	Tumor any site %	Tumor any site P
					Controls†					
0	189	108	57		141	74.6		174	92.1	
					Human serum fibronectin					
200	10	3	30	NS	2	20	<0.005	5	50	<0.0005
500	9	0	0	0.005	2	22	<0.005	2	22	<0.0001
1000	10	2	20	NS	0	0	<0.0001	2	20	<0.0001
					Mouse cellular fibronectin					
100	19	3	16	<0.001	6	32	<0.0005	13	68	<0.01
200	20	1	5	<0.0005	5	25	<0.0001	9	45	<0.0001
					Laminin					
100	9	5	56	NS	2	22	<0.005	8	89	NS
200	10	2	20	NS	2	20	<0.005	3	30	<0.0001
					RGDSPASSKP (synthetic decapeptide fragment of fibronectin)					
50	8	0	0	<0.001	3	38	NS	3	38	<0.0001
100	9	1	11	<0.05	1	11	<0.005	1	11	<0.0001
200	9	3	33	NS	1	11	<0.005	4	44	0.0001
					RGDS (synthetic tetrapeptide fragment of fibronectin)					
50	10	1	10	<0.05	4	40	NS	6	60	<0.01
100	10	0	0	<0.005	5	50	NS	8	80	NS
200	10	0	0	<0.005	2	20	<0.005	8	80	NS
400	11	1	9	<0.05	3	27	<0.005	9	82	NS
					Bovine fibrinogen					
100	10	2	20	NS	5	50	NS	8	80	NS
200	10	0	0	<0.01	4	40	NS	6	60	<0.01
400	9	1	11	<0.05	3	33	<0.05	5	56	<0.005
					KQAGDV (fibrinogen peptide)					
100	10	7	70	NS	4	40	NS	9	90	NS
200	10	2	20	NS	5	50	NS	6	60	<0.01
					EDTA (chelating agent)					
5 mM	10	4	40	NS	2	20	<0.005	5	50	<0.0005
10 mM	8	1	13	NS	2	25	<0.005	2	25	<0.0001
					Anti-α_5 antibody					
25	10	8	80	NS	5	50	NS	9	90	NS
					Anti-PECAM antibody					
25	7	6	86	NS	7	100	NS	7	100	NS

†Controls represent both historical as well as concurrent controls. They are presented together as the results were reproducible.

*P = significance level of difference in the frequency of tumor formation in the control versus treated mice, determined by Chi-square test with the Yates continuity correction factor.

wound sites have not been clearly identified, but it is known that several factors released during the immediate post-surgical phase are chemotactic to a variety of cells [2,3,34]. Once tumor cells arrive at the wound site, they interact with the extracellular matrix molecules for implantation.

The major extracellular matrix components that become available in the immediate postsurgical stage in-

TABLE V. Tumor Formation at Sites of Trauma in Relation to Timing of Tumor Injection

Post-op day	No. of mice	Mice with tumor: Lung n	Lung %	Surg. skin n	Surg. skin %	Surg. peritoneum n	Surg. peritoneum %	Surg. target n	Surg. target %	Tumor any site n	Tumor any site %
								Spleen			
0*	189	108	57	10	5	39	21	141	75	174	92
1	20	7	35	2	10	4	20	5	25	11	55
4	12	16	8	0	0	1	8	1	8	1	8
11	10	2	20	0	0	0	0	0	0	4	40
≥15	9	3	11	1	11	0	0	0	0	3	33
								Cecum			
0*	51	25	49	12	24	24	47	38	75	47	92
3	10	3	30	0	0	0	0	1	10	4	40
8	7	0	0	1	14	1	14	2	29	2	29
10	9	3	30	0	0	0	0	2	11	4	44
								Liver			
0*	240	129	54	23	10	77	32	107	45	203	85
1	34	22	65	4	12	6	18	10	29	28	85
7	5	4	80	0	0	0	0	1	20	4	80
≥10	23	23	100	0	0	0	0	0	0	23	100

*Controls (post-op day 0) represent both historical as well as concurrent controls. They are presented together, as the results were reproducible.

clude fibronectin, laminin, fibrinogen, collagen, and proteoglycans. These molecules have been demonstrated to play a pivotal role in tumor metastasis [35–39]. In addition to the extracellular matrix components, tumor cells may also interact with the endothelial cells and the platelets. Tumor cells interact with these targets via integrin, immunoglobulin, and cadherin types of receptors [40–43]. Once the cells attach to the target sites, they anchor firmly to the matrix so that they are not easily dislodged by irrigation with saline or water [44]. However, if the irrigation fluid also includes RGDS [45], which serves as an antiadhesive molecule, tumor implantation is prevented. These findings support the contention that the cells are likely to utilize RGDS containing matrix components for implantation.

The precise nature of the matrix molecules and the cell surface receptors used for implantation at wound sites are not known. Therefore, TA3Ha and TA3AD cell lines were examined for the expression of ICAM-1, PECAM, and the integrin receptor β_3 and β_1 subfamilies. ICAM-1 and PECAM were not detectable on either TA3Ha or TA3AD cells. The β_3 subfamily includes the receptors for fibrinogen, von Willebrand factor, vitronectin, and thrombospondin [46,47]. The β_1 has a broader selectivity than the β_3 subfamily. β_1 subunit in association with α_1, α_2, α_3, or α_6 mediates binding to collagen and/or laminin; with α_3, α_4, or α_5, it serves as fibronectin receptor; and with α_4 it is also a receptor for the endothelial cell adhesion molecules E-selectin or VCAM-1 (vascular cell adhesion molecule-1). α_v integrin subunit recognizes only vitronectin with β_1. It recognizes vitronectin and fibronectin when associated with β_5, and fibronectin, vitronectin, and fibrinogen when associated with β_3 [46,47].

β_3 integrin subunit was not demonstrable on TA3Ha cells with the monoclonal antibodies used. Thus, the possibility of vitronectin, von Willebrand factor, thrombospondin, and fibrinogen as the major ligands in a β_3-dependent manner is minimized. Among the α-subunits, α_v, is not expressed. Since it is a major receptor subunit for vitronectin, the roles of vitronectin in tumor implantation at wound sites is further diminished. The pattern of expression of these receptor subunits was identical in the TA3AD cells also.

The α_4 was not demonstrable on either TA3Ha or TA3AD. α_5 was demonstrable on TA3Ha, but not on TA3AD. In a panel of human tumor cell lines also a correlation was noted between the expression of α_5-subunit and the ability to implant at surgical sites in athymic nude mice [29]. These results strongly suggest an important role for fibronectin receptor in implantation of cells at surgical wound sites. The possible involvement of fibronectin receptor in wound implantation is further supported by blocking experiments. Pretreatment of TA3Ha cells with human serum fibronectin or mouse cellular fibronectin effectively prevented TA3Ha implantation at splenic wound site ($P < 0.05$). These results are identical to those found in the injured liver model (30,31). Similarly, the synthetic peptide fragments of fibronectin,

namely RGDSPASSKP or RGDS, also caused a significant ($P < 0.01$) reduction in splenic wound site implantation. These same synthetic deca- and tetrapeptides showed similar inhibitory effects on tumor implantation at sites of surgery in the liver [30,31], bone [23], and the peritoneum [3]. It appears that the tumor cells may use similar mechanisms for implantation at wound sites across different organs. An important, although not an exclusive mechanism for tumor implantation at surgical sites involves fibronectin and its receptor β_1/α_5.

The strength of the conclusion regarding the role of the fibronectin receptor is, however, tempered by our inability to completely block TA3Ha implantation at surgical sites by anti-α_5 antibodies. We presume that incomplete blockage of tumor implantation may be because the antibodies used are partial blocking antibodies, and the concentration of the antibodies used is 25 μg/ml. This issue will be resolved in our future experiments by modulating the expression of α_5-receptor by introducing sense and antisense expression vectors into the TA3AD and TA3Ha cells, respectively. Thus, until these data are collected, the conclusion on the role of α_5-integrin subunit in tumor implantation is considered less than definitive.

In summary, tumor cells implant at injured intra-abdominal sites with little regard to the specific organ that is injured. The tumor cells able to implant at wound sites also are able to form spontaneous distant metastasis and local recurrence. Conversely, those that fail to implant at wound sites also fail to form local recurrence. Tumor implantation at wound sites and the formation of local recurrence correlate with the expression of α_5/β_1 molecules. Tumor implantation at surgical sites appear to utilize similar mechanisms independent of the organs involved.

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